Synergistic effects on gene delivery - co-formulation of small disulfide-linked dendritic polycations with Lipofectamine 2000 (TM)

Synergistic effects on gene delivery – co-formulation of small disulfide-linked dendritic polycations with Lipofectamine 2000™†

John G. Hardy [a], **Christine S. Love** [a], **Nathan P. Gabrielson** [b], **Daniel W. Pack** [b] **and David K. Smith** *[a]

[a]*Department of Chemistry, University of York, Heslington, York, YO10 5DD, UK. E-mail: dks3@york.ac.uk; Fax: +44 1904 432516*

[b]*Department of Chemical and Biomolecular Engineering, University of Illinois at Urbana Champaign, Urbana, IL 61801, USA*

This paper describes the application of gene delivery vectors based on connecting together two well-defined low-generation poly(L-lysine) (PLL) dendrons using a disulfide-containing linker unit. We report that the transfection ability of these vectors in their own right is relatively low, because the low-generation number limits the endosomal buffering capacity. Importantly, however, we demonstrate that when applied in combination with Lipofectamine 2000™, a vector from the cationic lipid family, these small cationic additives significantly enhance the levels of gene delivery (up to four-fold). Notably, the cationic additives have no effect on the levels of transfection observed with a cationic polymer, such as DEAE dextran. We therefore argue that the synergistic effects observed with Lipofectamine 2000™ arise as a result of combining the delivery advantages of two different classes of vector within a single formulation, with our dendritic additives providing a degree of pH buffering within the endosome. As such, the data we present indicate that small dendritic structures, although previously largely overlooked for gene delivery owing to their inability to transfect in their own right, may actually be useful well-defined additives to well-established vector systems in order to enhance the gene delivery payload.

Introduction

The development of effective vectors for the delivery of genetic materials such as DNA and/or siRNA into cells is one of the most important targets of medicinal chemists – success

would have a major impact across a wide range of different diseases.[1] Recently, attention has begun to increasingly focus on the potential of non-viral vectors in gene delivery,[2] in particular, because they have the opportunity to avoid problems associated with viral vectors such as immunogenicity and other side effects.[3] Two classes of vector have seen particularly intensive development – cationic polymers[4] and cationic lipids.[5] Cationic polymers achieve DNA binding and delivery owing to their large number of cationic sites which can effectively bind polyanionic nucleic acid – however, polycations are known to be toxic *in vivo*, leading to hemolysis and other unwanted side effects. Cationic lipids, on the other hand, bind DNA as they assemble multiple positively charged units in a non-covalent manner. However, it is important that the payload of genetic material be optimised with this class of vector in order to maximise the effect of gene delivery. A number of non-viral vectors have been effectively commercialised and have seen widespread *in vitro* use. As yet, however, in spite of extensive work and a number of clinical trials,[2] non-viral vectors have not yet entered into routine clinical use. In particular, in the process of vector development, it is vital to minimise toxicity/immunogenicity and maximise the genetic payload.

The use of dendritic gene delivery agents has been of considerable interest.[6] For example, Superfect™ is a fractured poly(amidoamine) (PAMAM) dendrimer developed by the groups of Szoka and Tomalia.[7]Poly(L-lysine) (PLL) dendrimers and dendrons[8] have also seen considerable development. Niidome and co-workers systematically investigated dendritic PLL and reported that high generations (*e.g.* 5th and 6th generation) were required for effective transfection to be observed.[9] Park and co-workers have made a number of synthetic modifications to lysine-derived dendritic architectures.[10] For example, they generated ABA-type block co-polymer dendrons in which the A groups were dendritic lysine units, whilst the B group was a linker, such as poly(ethylene oxide) (PEG). AB-type derivatives have also been investigated in which a dendron based on L-lysine is attached to other units. In key work, Florence, Toth and co-workers appended hydrophobic units at the focal point, in order to enhance the self-assembly characteristics of the dendritic architecture.[11] Such structures were tested both *in vitro* and *in vivo* and were demonstrated to show good levels of transfection. Vlasov and co-workers have also employed hydrophobic modifications of dendritic PLL to enhance levels of transfection.[12] Cao and co-workers used a degradable poly(L-lactide) block as the linker between dendritic units in an ABA structure,

however, they only applied second-generation lysine units, and the transfection observed was therefore only very modest.[13]

We have recently been interested in investigating the ability of relatively small and/or degradable dendrons to bind DNA and deliver genetic material *in vitro*.[14] We argue that such systems should exhibit lower toxicities than their larger polymeric analogues as they will be less likely to persist in cells after transfection has taken place. However, in their own right, as found in numerous studies, if cationic polymers are too small, they have limited ability to transfect, because there are insufficient amine groups to cause pH buffering within the endosome – as such, the vector/DNA complex is unable to effectively escape from endosomes and gene delivery is ineffective. There have been a handful of reports in which cationic lipids have been mixed with cationic polymers giving rise to significant enhancements in gene delivery.[15] We have therefore become interested in exploring synergistic effects using our relatively small dendritic architectures by combining aspects of both cationic lipid and cationic polymer characteristics.[16] These two main classes of non-viral vector have different delivery modes within the cell. Cationic lipids can escape from endosomes*via* lipidic destabilisation of the endosomal membrane,[17] whilst cationic polymers achieve endosomal rupture by pH-buffering effects.[18] We therefore reason that these two effects can act in concert to amplify delivery significantly over what might be expected based on data from either vector taken individually.

In this paper we explore the use of small dendritic ABA-type vectors based on dendritic PLL connected together using a biodegradable disulfide linkage (Fig. 1). We only investigate relatively low-generations of dendrimer in order to minimise toxicity of the polycationic surface and to ensure that all of the compounds are well-defined, monodisperse architectures, important in biomedical applications. Furthermore, the choice of the disulfide linkage should mean that in later *in vivo* studies, this vector would be expected to break into two even smaller, less highly positively charged (and hence less toxic) units.[19] It was our intention to explore the ability of these small cationic dendrimers to transfect, and in particular to determine whether synergistic effects on gene delivery would be observed when these vectors were applied together with a well-established cationic lipid or polymer vector – commercially available Lipofectamine 2000™ (cationic lipid) and DEAE dextran (cationic polymer) were chosen for this study.

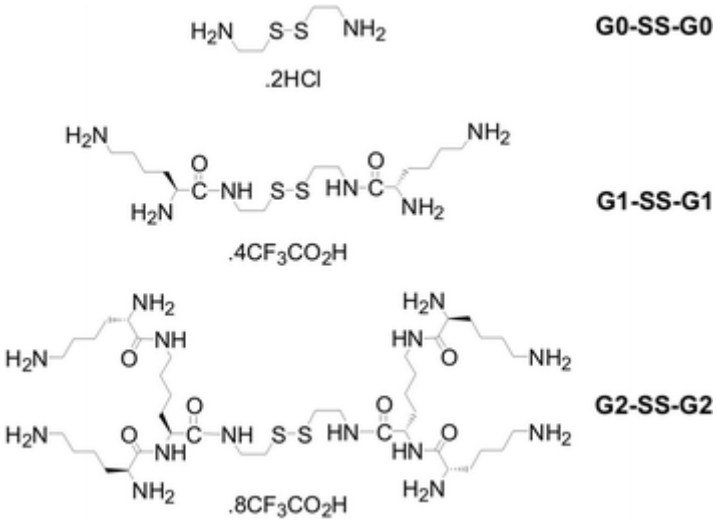

Fig. 1 Cationic additives for gene delivery investigated in this paper.

Results and discussion

Synthesis of cationic dendrimers

The syntheses of the vectors used in this study (in Boc-protected form) were previously reported and the dendritic materials were applied in materials science.[20] The Boc-protecting groups were readily removed in quantitative yield by adding trifluoroacetic acid in dichloromethane to the dendrimers, which after evaporation of the solvent provided the target amine-surfaced cationic dendrimers, **G1-SS-G1** and **G2-SS-G2**, as highly water-soluble trifluoroacetate salts (Fig. 1). Compound **G0-SS-G0** was commercially available as its hydrochloride salt. We then went on to investigate the ability of these dendrimers to bind DNA.

Gel retardation studies

Gel electrophoresis provides an effective means of investigating the interaction between cationic polymers and plasmid DNA. The formation of polyplexes is observed as a reduction of mobility of the plasmid DNA (Fig. 2). In these studies, DNA was mixed with increasing amounts of polyamine in order to determine the ability of the polyamine to form complexes with the DNA. As expected, the **G0-SS-G0** model compound (cystamine dihydrochloride) failed to show any ability to retard the migration of DNA, even at mass ratios of 1 : 100

(DNA:amine). This is not surprising given the fact this compound only contains two protonatable nitrogen atoms. Normally, tetraamines such as spermine and spermidine are required to achieve moderate levels of DNA binding – such systems are present in eukaryotic cells where they play an active DNA binding role. In contrast, some retardation was observed with tetraamine**G1-SS-G1** at reasonably high mass loadings, and this effect was enhanced for octaamine **G2-SS-G2**, which began to retard plasmid DNA even at wt/wt ratios as low as 1 : 1 (Fig. 2). However, the levels of binding observed were much lower than those previously recorded for our spermine-derived dendrons.[14,16] This reflects the expected relatively weak DNA binding ability of these low-generation lysine dendrimers.[9,13]

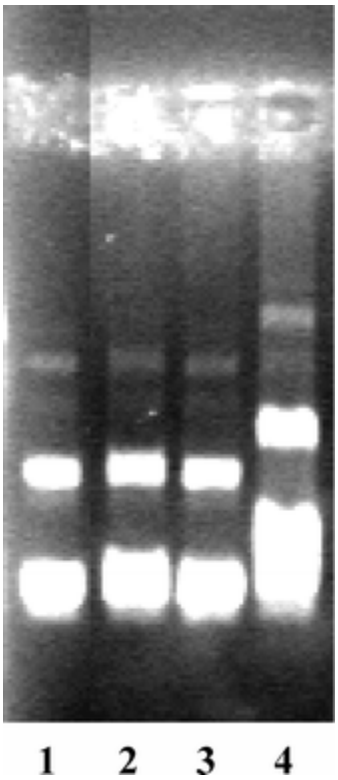

Fig. 2 Agarose gel electrophoresis of plasmid DNA (250 ng per lane). Lane 1: Plasmid DNA. Lane 2: Plasmid DNA + **G0-SS-G0** (250 ng). Lane 3: Plasmid DNA + **G1-SS-G1** (250 ng). Lane 4: Plasmid DNA + **G2-SS-G2** (250 ng).

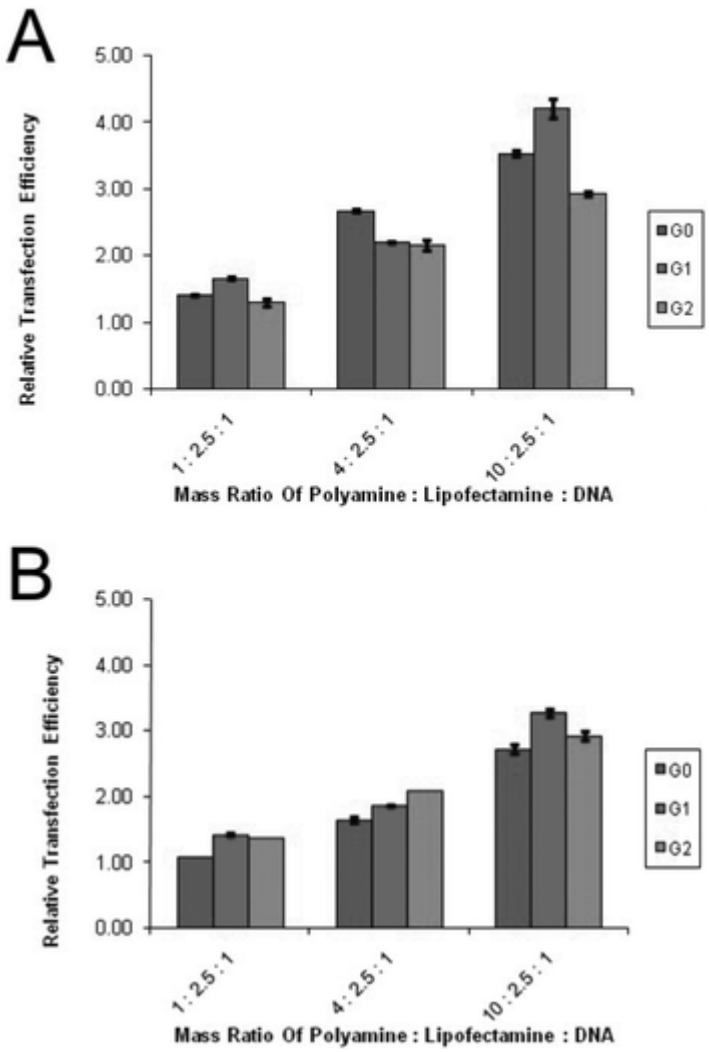

Fig. 3 Relative transfection efficiencies of lipopolyplexes in (A) C2C12 and (B) MDA-MB-231 cells. Transfection efficiency was measured as luciferase expression normalized by total cellular protein (average normalized RLU mg^{-1} of protein), and subsequently normalized against the transfection efficiency of Lipofectamine 2000™/DNA complexes to give the relative transfection efficiency. (*N* = 6, error bars represent standard deviation).

Gene transfection

We then investigated the efficacy of the polyamines (**G0-SS-G0**, **G1-SS-G1** and **G2-SS-G2**) in gene delivery to human breast carcinoma cells (MDA-MB-231) and murine myoblasts (C2C12). Both cell lines were transfected *in vitro* with 1 μg of plasmid DNA per 10^5cells. In each case, the DNA was complexed with varying amounts of the different amines. Gene transfection efficiency was measured as luciferase enzyme activity and normalized to total cell protein.

Initially, the polyamines were investigated in their own right to determine their ability to transfect DNA into cells and allow expression of luciferase, however no measurable transfection could be observed in any case. This was not surprising, as the DNA binding of these systems was relatively weak, and it was previously known that low-generation lysine-derived systems were ineffective.[9,13]

We have been interested in synergistic effects on gene delivery[16] and therefore investigated co-formulation of the polyamines with a cationic lipid (Lipofectamine 2000™) and a cationic polymer (DEAE dextran). In the case of polymeric DEAE dextran, there was no advantage in co-formulating the amines with the polymer:DNA polypoplex. However, on mixing the amine with the Lipofectamine 2000™:DNA lipoplex, significant improvements in the gene delivery payload were observed. Interestingly all of the amines were observed to improve the measurable gene expression over the range of polyamine:Lipofectamine 2000™:DNA mass ratios investigated (1 : 2.5 : 1, 4 : 2.5 : 1 and 10 : 2.5 : 1), with the general trend that the transfection efficiency increased as the quantity of polyamine increased. Similar trends were observed for both cell lines, although the transfection into murine myoblasts (C2C12) was slightly higher than that for the human breast carcinoma cells (MDA-MB-231). We argue that the amine additives increase the observed levels of transfection because they weakly associate with the DNA lipoplex *via* electrostatic interactions, but are importantly able to assist with the process of lipoplex endosomal escape. It is well known that polyamines assist with endosomal escape because of their ability to buffer pH changes, leading to endosomal swelling and rupture.[18] We propose that this mechanism operates in this case, and that the amines therefore have a synergistic effect on the delivery of DNA. This proposal is supported by the observation that the presence of the cationic amine additive does not enhance the transfection observed with DEAE dextran. This vector is, in its own right, a cationic polymer with its own buffering capacity, and would not be expected to benefit from the presence of additives capable of performing this role. It was perhaps a bit

surprising that the number of amines present on the cationic additive appeared to only have a limited effect on the transfection enhancement –**G1-SS-G1** was only marginally more effective than **G0-SS-G0**, and **G2-SS-G2** had lower/similar efficiency. To study this further we investigated vector cytotoxicity.

Cytotoxicity determination

We determined cytotoxicities of the dendrons using an XTT assay. It is well-known that polycationic molecules damage cell membranes as a result of the electrostatic attraction of polycations to the plasma membrane. On the other hand, neutral and anionic polymers cause minimal damage to cellular membranes. This toxicity of cationic polymers and dendrimers has been one of the major drawbacks of their development for *in vivo*gene delivery applications. We hypothesised that the low-generation systems employed as additives in this work should be relatively non-toxic, and hence their application should perhaps be more viable. Human breast carcinoma cells (MDA-MB-231) and murine myoblasts (C2C12) were exposed to various conditions (*i.e.*, polyamine, polyamine + DNA, polyamine + DNA + Lipofectamine 2000™) for 4 h, and metabolic activity was assayed 20 h later. The results of these studies are collected in Table 1.

Table 1 Cytotoxicity of the polyamines**G0-SS-G0**, **G1-SS-G1**, **G2-SS-G2** and poly(ethyleneimine) (PEI, 25 kDa) in murine myoblast C2C12 and human breast carcinoma MDA-MB-231 cells

		Metabolic activity (rel. to 1.000)			
Cell line	**Conditions**	**G0**	**G1**	**G2**	**PEI**
C2C12	Polyamine alone	0.810	1.091	1.058	0.705
C2C12	Polyamine + DNA	0.990	1.053	1.047	0.808
C2C12	Polyamine/DNA + lipofectamine	0.898	0.636	0.714	0.623
MDA-MB-231	Polyamine alone	1.027	1.037	1.072	0.769
MDA-MB-231	Polyamine + DNA	0.983	1.153	1.077	0.954
MDA-MB-231	Polyamine/DNA + lipofectamine	1.274	0.890	0.837	0.423

No reduction in metabolic activity was observed when the polyamine alone was added at a concentration of 1 μg per 1000 cells. The one exception to this was when a polyamine control (25 kDa poly(ethyleneimine), PEI) was employed, in which case the observed metabolic activity was reduced by 30% for the C2C12 cells. This is important as it indicates that our low-generation dendritic amines are, as anticipated, significantly less cytotoxic than a typically employed cationic polymer, PEI. Similarly, when a combination of polyamine and DNA was added to the cells (0.2 μg DNA and 1 μg polyamine per 1000 cells), our dendrons (G1 and G2) pleasingly had no observable cytotoxicity. Once again, 25 kDa PEI reduced metabolic activity, by 20% for the C2C12 cells – demonstrating the greater toxicity of larger polymers.

We then tested cytotoxicity under our transfection conditions (*i.e.*, in the presence of lipofectamine 2000™ and DNA). Under these conditions, some toxicity was observed. At a relatively high 10:2.5 : 1 mass ratio (0.1 μg polyamine, 0.025 μg Lipofectamine 2000™ and 0.01 μg DNA per 1000 cells), **G1-SS-G1** and **G2-SS-G2** caused a reduction in metabolic activity of *ca.* 25–40% in C2C12 cells and 10–20% in MDA-MB-231 cells. The effect of **G0-SS-G0** on metabolic activity was less significant than that of the dendritic analogues – this could either be due to its lower cationic character, or more probably because it was used as the hydrochloride salt rather than the trifluoroacetate, *i.e.* the presence of the trifluoroacetate counterion may well have led to these observed toxicities.[21] We propose that the toxicity data may explain why the transfection enhancements observed with **G1-SS-G1** and **G2-SS-G2** were perhaps less than might have been expected based on simple buffering capacity alone (in comparison with **G0-SS-G0**) and that toxicity may to some extent be limiting the levels of transfection observed with these branched systems. It should be noted that these cytotoxicities are measured at relatively high cation loading – lower levels can be used for transfection and still give rise to synergistic effects.

Conclusions

In conclusion, this study supports the hypothesis that cationic additives can enhance the transfection levels observed with cationic lipids. This hybrid vector approach has significant potential in enhancing gene delivery payloads. Interestingly, even non-polymeric and low-generation dendritic cations, which have no transfection effect in their own right, have

significant positive effects on transfection ability, demonstrating how the advantages of cationic 'polymers' can be combined with those of cationic lipids. Furthermore, we note that the disulfide design of our additives should make them prone to eventual cellular degradation,[19] breaking them down into smaller units, and potentially limiting longer-term toxic effects associated with vector accumulation within transfected cells.[22] Further work on these vectors would focus on modifying the counter-anion to monitor its effect on toxicity, identifying cellular degradation products, as well as testing the transfection potential of these hybrid systems under more challenging conditions.

Acknowledgements

We gratefully acknowledge financial support from the EPSRC and Syngenta (J.G.H. and C.S.L.) and a generous scholarship from the Worldwide Universities Network (WUN) which allowed J.G.H. to visit the laboratories in Illinois.

References

1. M. L. Edelstein, M. R. Abedi and J. Wixon, *J. Gene Med.*, 2007, **9**, 833–842 .
2. K. Kodama, Y. Katayama, Y. Shoji and H. Nakashima, *Curr. Med. Chem.*, 2006, **13**, 2155–2161 .
3. R. Tomanin and M. Scarpa, *Curr. Gene Ther.*, 2004, **4**, 357–372 .
4. S. Y. Wong, J. M. Pelet and D. Putnam, *Prog. Polym. Sci.*, 2007, **32**, 799–837 .
5. P. P. Karmali and A. Chaudhuri, *Med. Res. Rev.*, 2007, **27**, 696–722 .
6. (*a*) C. Dufes, I. F. Uchegbu and A. G. Schatzlein, *Adv. Drug Delivery Rev.*, 2005, **57**, 2177–2202 ; (*b*) M. Guillot-Nieckowski, S. Eisler and F. Diederich, *New J. Chem.*, 2007, **31**, 1111–1127 ; (*c*) D. K. Smith, *Curr. Top. Med. Chem.*, 2008, **8**, 1187–1203 .
7. (*a*) A. Bielinska, J. F. Kukowska-Latallo, J. Johnson, D. A. Tomalia and J. R. Baker, *Nucleic Acids Res.*, 1996, **24**, 2176–2182 (*b*) M. P. Turunen, M. O. Hitunen, M. Ruponen, L. Virkamäki, F. C. Szoka, A. Urtti and S. Ylä Herttuala, *Gene Ther.*, 1999, **6**, 6–11.
8. (*a*) R. G. Denkewalter, J. F. Kolc and W. J. Lukasavage, *US Pat.*, 4 360 646, 1979 ; (*b*) R. G. Denkewalter, J. F. Kolc and W. J. Lukasavage, *US Pat.*, 4 289 872, 1981; (*c*) R. G. Denkewalter, J. F. Kolc and W. J. Lukasavage, *US Pat.*, 4 410 688, 1983 .

9. (*a*) M. Ohsaki, T. Okuda, A. Wada, T. Hirayama, T. Niidome and H. Aoyagi, *Bioconjugate Chem.*, 2002, **13**, 510–517 ; (*b*) T. Okuda, S. Kidoaki, M. Ohsaki, Y. Koyama, K. Yoshikawa, T. Niidome and H. Aoyagi, *Org. Biomol. Chem.*, 2003, **1**, 1270–1273 ; (*c*) M. Yamagata, T. Kawano, K. Shiba, T. Mori, Y. Katayama and T. Niidome, *Bioorg. Med. Chem.*, 2007, **15**, 526–532 .
10. (*a*) J. S. Choi, E. J. Lee, Y. H. Choi, Y. J. Jeong and J. S. Park, *Bioconjugate Chem.*, 1999, **10**, 62–65 ; (*b*) J. S. Choi, D. K. Joo, C. H. Kim, K. Kim and J. S. Park, *J. Am. Chem. Soc.*, 2000, **122**, 474–480 ; (*c*) K. D. Eom, S. M. Park, H. D. Tran, M. S. Kim, R. N. Yu and H. Yoo, *Pharm. Res.*, 2007, **24**, 1581–1589 .
11. (*a*) I. Toth, T. Sakthivel, A. F. Wilderspin, H. Bayele, M. O'Donnell, D. J. Perry, K. J. Pasi, C. A. Lee and A. T. Florence, *STP Pharma Sci.*, 1999, **9**, 93–99 ; (*b*) D. S. Shah, T. Sakthivel, I. Toth, A. T. Florence and A. F. Wilderspin, *Int. J. Pharm.*, 2000, **208**, 41–48 ; (*c*) K. T. Al-Jamal, C. Ramaswamy, B. Singh and A. T. Florence, *J. Drug Delivery Sci. Technol.*, 2005, **15**, 11–18 ; (*d*) H. K. Bayele, T. Sakthivel, M. O'Donell, K. J. Pasi, A. F. Wilderspin, C. A. Lee, I. Toth and A. T. Florence, *J. Pharm. Sci.*, 2005, **94**, 446–457 (*e*) R. J. Marano, I. Toth, N. Wimmer, M. Brankov and P. E. Rakoczy, *Gene Ther.*, 2005, **12**, 1544–1550 .
12. (*a*) G. P. Vlasov, V. I. Korol'kov, G. A. Pankova, I. I. Tarasenko, A. N. Baranov, P. B. Glazkov, A. V. Kiselev, O. V. Ostapenko, E. A. Lesina and V. S. Baranov, *Russ. J. Bioorg. Chem.*, 2004, **30**, 12–20 (*b*) G. P. Vlasov, E. A. Lesina, V. I. Korol'kov, I. A. Gur'yanov, N. V. Bayanova, A. N. Baranov, A. V. Kiselev and V. S. Baranov, *Russ. J. Bioorg. Chem.*, 2005, **31**, 153–159 ; (*c*) A. V. Kiselev, P. L. Il'ina, A. A. Egorova, A. N. Baranov, I. A. Guryanov, N. V. Bayanova, I. I. Tarasenko, E. A. Lesina, G. P. Vlasov and V. S. Baranov, *Russ. J. Genet.*, 2007, **43**, 593–600 .
13. Y. Li, L. Cui, Q. Li, L. Jia, Y. Xu, Q. Fang and A. Cao, *Biomacromolecules*, 2007, **8**, 1409–1416.
14. (*a*) M. A. Kostiainen, J. G. Hardy and D. K. Smith, *Angew. Chem., Int. Ed.*, 2005, **44**, 2556–2559 ; (*b*) J. G. Hardy, M. A. Kostiainen, D. K. Smith, N. P. Gabrielson and D. W. Pack, *Bioconjugate Chem.*, 2006, **17**, 172–178 ; (*c*) M. A. Kostiainen, G. R. Szilvay, D. K. Smith, M. B. Linder and O. Ikkala, *Angew. Chem., Int. Ed.*, 2006, **45**, 3538–3542 ; (*d*) M. A. Kostiainen, G. R. Szilvay, J. Lehtinen, D. K. Smith, M. B. Linder, A. Urtti and

O. Ikkala, *ACS Nano*, 2007, **1**, 103–113 ; (*e*) M. A. Kostianinen, D. K. Smith and O. Ikkala, *Angew. Chem., Int. Ed.*, 2007, **46**, 7600–7604 .

15. (*a*) X. Gao and L. Huang, *Biochemistry*, 1996, **35**, 1027–1036 ; (*b*) F. L. Sorgi, S. Bhattacharya and L. Huang, *Gene Ther.*, 1997, **4**, 961–968 ; (*c*) J. F. Kukowska-Latallo, C. Chen, J. Eichman, A. U. Bielinska and J. R. Baker, *Biochem. Biophys. Res. Commun.*, 1999, **264**, 253–261 (*d*) P. Lampela, P. Soininen, A. Urtti, P. Männistö and A. Raasmaja, *Int. J. Pharm.*, 2004, **270**, 175–184 (*e*) T. H. Kim, S. I. Kim, T. Akaike and C. S. Cho, *J. Controlled Release*, 2005, **105**, 354–366 ; (*f*) J. Pelisek, L. Gaedtkel, J. De Rouchey, G. F. Walkerl, S. Niko and E. Wagner, *J. Gene Med.*, 2006, **8**, 186–197 (*g*) A. Golda, J. Pelisek, R. Klocke, M. G. Engelmann, P. H. Rolland, C. Mekkaoui and S. Nikol, *J. Vasc. Res.*, 2007, **44**, 273–283 , (*h*) J.-L. Chen, H. Wang, J.-Q. Gao, H.-L. Chen and W.-Q. Liang, *Int. J. Pharm.*, 2007, **343**, 255–261 (*i*) L. Garcia, M. Brunuales, N. Duzgunes and C. T. de Ilarduya, *Eur. J. Pharm. Biopharm.*, 2007, **67**, 58–66 (*j*) L. Brito, S. Little, R. Langer and M. Amiji, *Biomacromolecules*, 2008, **9**, 1179–1187 .
16. S. P. Jones, N. P. Gabrielson, D. W. Pack and D. K. Smith, *Chem. Commun.*, 2008, 4700–4702 .
17. I. S. Zuhorn, J. B. F. N. Engberts and D. Hoekstra, *Eur. Biophys. J.*, 2007, **36**, 349–362 .
18. N. D. Sonawane, F. C. Szoka and A. S. Verkman, *J. Biol. Chem.*, 2003, **278**, 44826–44831 .
19. (*a*) M. A. Gosselin, W. Guo and R. J. Lee, *Bioconjugate Chem.*, 2002, **13**, 1044–1053 ; (*b*) D. Oupicky, A. L. Parker and L. W. Seymour, *J. Am. Chem. Soc.*, 2002, **124**, 8–9 ; (*c*) M. L. Read, S. Singh, Z. Ahmed, M. Stevenson, S. S. Briggs, D. Oupicky, L. B. Barrett, R. Spice, M. Kendall, M. Berry, J. A. Preece, A. Logan and L. W. Seymour, *Nucleic Acids Res.*, 2005, **33**, e86 ; (*d*) C. Pichon, E. LeCam, B. Guerin, D. Coulad, E. Delain and P. Midoux, *Bioconjugate Chem.*, 2002, **13**, 76–82; (*e*) Y. Lee, H. Mo, H. Koo, J.-Y. Park, M. Y. Cho, G. -w. Jin and J. -S. Park, *Bioconjugate Chem.*, 2007, **18**, 13–18 ; (*f*) C. Lin, Z. Zhong, M. C. Lok, X. Jiang, W. E. Hennik, J. Feijen and J. F. J. Engbersen, *Bioconjugate Chem.*, 2007, **18**, 138–145 .
20. (*a*) C. S. Love, V. Chechik, D. K. Smith and C. Brennan, *J. Mater. Chem.*, 2004, **14**, 919–923 ; (*b*) C. S. Love, A. R. Hirst, V. Chechik, D. K. Smith, I. Ashworth and C.

Brennan, *Langmuir*, 2004, **20**, 6580–6585 ; (*c*) C. S. Love, V. Chechik, D. K. Smith, K. Wilson, I. Ashworth and C. Brennan, *Chem. Commun.*, 2005, 1971–1973 .

21. M. J. Little, N. Aubry, M.-E. Beaudoin, N. Goudreau and S. R. LaPlante, *J. Pharm. Biomed. Anal.*, 2007, **43**, 1324–1330 .
22. J.-H. S. Kuo and Y. -L. Lin, *J. Biotechnol.*, 2007, **129**, 383–390 .